The Interplay between HTLV-1 Viral Factors, Tax and HBZ, During T-cell Transformation

Honors Research Thesis

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) is a tumorigenic retrovirus that infects an estimated 15-20 million people worldwide. HTLV-1 is responsible for an aggressive T-cell malignancy termed adult T-cell leukemia/lymphoma (ATLL). The incidence of disease associated with HTLV-1 infection is 2-6% over the lifetime of an infected individual, with symptoms taking up to 3-4 decades to present. Despite the long clinical latency period, HLTV-1-associated malignancies are chemotherapy resistant and the median survival time is <1 year. The detailed mechanism of how HTLV-1 transforms cells is still unknown, but several studies have indicated that two viral factors, Tax and HBZ, are individually linked to oncogenic transformation. However, the interplay between Tax and HBZ in the transformation process is unknown. Herein, we investigated the relationship between Tax and HBZ using a dual-inducible lentiviral expression system. Tax and HBZ cDNAs were cloned into the cumate-inducible and doxycycline-inducible expression vectors, respectively. These vectors were transduced into CTLL-2 cells and stable cellular clones were selected using drug resistance and single cell dilution. Clones that responded best to drug induction (as measured by mRNA levels and protein functionality) were selected for further experiments. Using the established CTLL-2 T-cell transformation assay, we sought to determine whether the presence of HBZ and Tax drives these cells from an IL-2-dependent to independent growth, a characteristic indicative of cellular transformation. Our results indicate that both Tax and HBZ enhance transformation. Ultimately, our work will provide better insight into the transformation landscape of T-cells in humans and the molecular pathogenesis of ATLL development.

INTRODUCTION

Human T-cell leukemia virus type 1 (HTLV-1) is a retrovirus, notable for its ability to cause adult T-cell leukemia/lymphoma (ATLL) in a small percentage of infected individuals after a long clinical latency period of 3-4 decades (Green et al. 2010). Specifically, ATLL is a disease in which T-cells of the immune system (primarily T helper cells) become cancerous. The exact mechanism of how this occurs is still unknown, but several key studies from our lab and others have suggested the involvement of two viral factors in this process: Tax and HBZ.

Tax is a viral transcriptional activator expressed early in viral infection (Green et al. 2010). This viral protein activates transcription of the viral genome and various cellular pathways involved in cellular proliferation. Previous published work has long established Tax as a viral oncogene - transforming cells through a variety of mechanisms including chromosomal instability (Jin et al. 1998), down-regulation of DNA repair mechanisms (Jeang et al. 1990), activation of cyclin-dependent kinases (Haller et al. 2000), deregulation of the NF-κB (Iha et al. 2003) and Akt signaling pathways (Peloponese et al. 2006), and silencing of the tumor suppressor protein p53 (Pise-Masison et al. 2000). It is also important to note that Tax serves as the primary viral antigen which is recognized by the host's immune system. Quite often, genetic and epigenetic inactivation of the Tax gene through mutations, promoter hypermethylation, and promoter deletion arise as the disease progresses to promote continual viral persistence (Matsuoka et al. 2007).

HTLV-1 basic leucine zipper factor (HBZ) is a modulator of both viral and cellular gene expression (Green et al. 2010). HBZ is also the only protein encoded on the

antisense genome strand/3' end of the provirus (Figure 1) (Matsuoka et al. 2007). Tax initially activates the transcription of HBZ, but the expression of Tax and HBZ are inversely proportional due to HBZ's ability to enhance the expression of PDLIM-2, a protein that promotes the degradation of Tax. HBZ also competes with Tax for basal cellular transcription factors such as CREB and p300 (Green et al. 2010). This is viewed as a viral adaptation for survival since Tax is highly immunogenic. Interestingly, in approximately 60% of ATLL cases, Tax is absent. However, *hbz* RNA is found in every ATLL patient and HTLV-1-infected cell line (Green et al. 2010). This is because the promoter driving *hbz* expression, the 3′ long terminal repeat (LTR), remains intact and is unmethylated at all stages of ATLL development, suggesting that the 3′ LTR has a critical role in maintenance of HTLV-1-mediated disease (Matsuoka et al. 2007). In summary, HTLV-1 has evolved where after Tax has initiated transformation, it is highly downregulated or even deleted to avoid detection by the immune system. There are currently no known HBZ specific cytotoxic T lymphocytes. Studies have shown that HBZ is not only a transcriptional activator, but is also important for the persistence of viral infection in late stages of ATLL development since HBZ knockout virus show significantly reduced tumor formation in animals (Green et al. 2010). Other studies have also suggested that *hbz* RNA, and not the HBZ protein, promotes T-cell proliferation due to RNA secondary structure (Matsuoka et al. 2007).

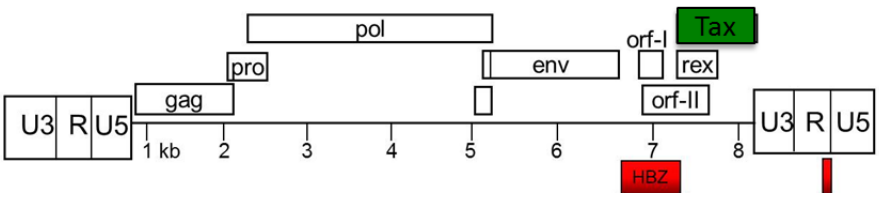

Figure 1. Genomic map of the HTLV-1 provirus. Tax is transcribed from the sense strand of the provirus along with many other structural, enzymatic, and accessory viral genes. HBZ is the only gene transcribed from the antisense genome stand.

Problem identification and justification

Overall, there is well-established evidence suggesting Tax and HBZ are individually linked to oncogenic transformation induced by HTLV-1. However, it is unclear if a relationship between Tax and HBZ in this transformation process occurs. The current theory within the field is that Tax initiates the transformation process, while HBZ provides the maintenance function. The purpose of this study is to gain a better understanding of the interplay between Tax and HBZ during T-cell transformation. Tax and HBZ are both expressed concurrently in a newly infected cell and therefore can affect the activities of each other. Altogether, our work will lead to better insight into the HTLV-1-mediated transformation process of T-cells and provide a better understanding towards therapies and treatments for ATLL. We hypothesize that the interplay between Tax and HBZ will enhance T-cell transformation and ultimately facilitate HTLV-1 pathogenesis.

MATERIALS AND METHODS

The relationship between Tax and HBZ was investigated by using a dual-inducible expression system in the IL-2-dependent murine T-cell line, CTLL-2. Tax and HBZ cDNAs were cloned into cumate-inducible and doxycycline-inducible expression vectors, respectively.

Overview of the Tet-ON HBZ inducible system

In the presence of doxycycline, Tet-On 3G serves as a transactivator protein driving the transcription of *hbz* RNA (Figure 2). This induction system is useful in mammalian tissue culture because pTRE3GV lacks binding sites for endogenous

transcription factors so it is virtually silent in the absence of doxycycline. Expression levels can be regulated following the addition of varying concentrations of drug.

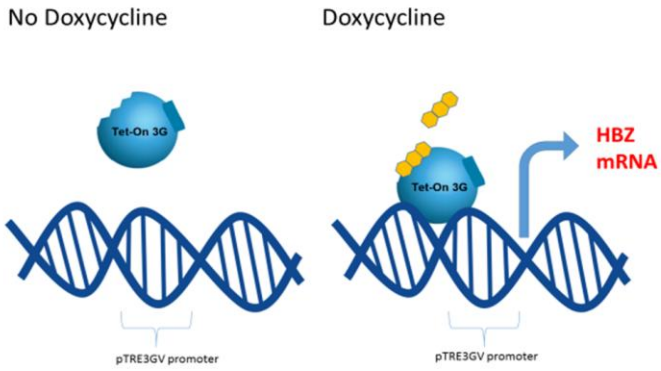

Figure 2. Tet-ON HBZ inducible system. In the presence of doxycycline, the transactivator protein Tet-ON 3G binds to the pTRE3GV promoter and drives transcription of HBZ mRNA. (Figure modified from Clontech).

Overview of the cumate Tax inducible system

The CymR repressor binds to the cumate operator sequences with high affinity preventing any transcription of downstream genes (Figure 3). However, repression is alleviated through the addition of Cumate (a non-toxic, small molecule that binds to CymR) which leads to the transcription of tax mRNA. Similar to the Tet system, expression levels can be regulated following the addition of variable concentrations of cumate.

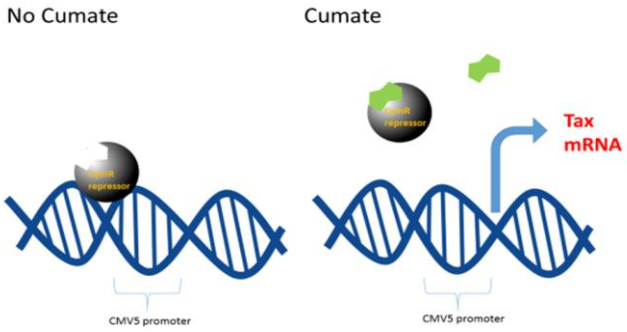

Figure 3. Cumate Tax inducible system. In the presence of cumate, the CymR repressor is alleviated from the CMV5 promoter and allows transcription of Tax mRNA. (Figure modified from Clontech).

Construction of the stable dual-inducible CTLL-2 cell line

Once successful cloning of Tax and HBZ cDNAs into the dual-inducible expression systems was completed, these lentiviral plasmids were integrated into the genome of the IL-2-dependent murine T-cell line, CTLL-2. This process first involves the transfection of lentiviral plasmids containing the two dual-inducible vectors, along with retroviral packaging components, into the adherent endothelial cell line, 293T (Figure 4). The cellular machineries of these cells then package the lentiviral components into

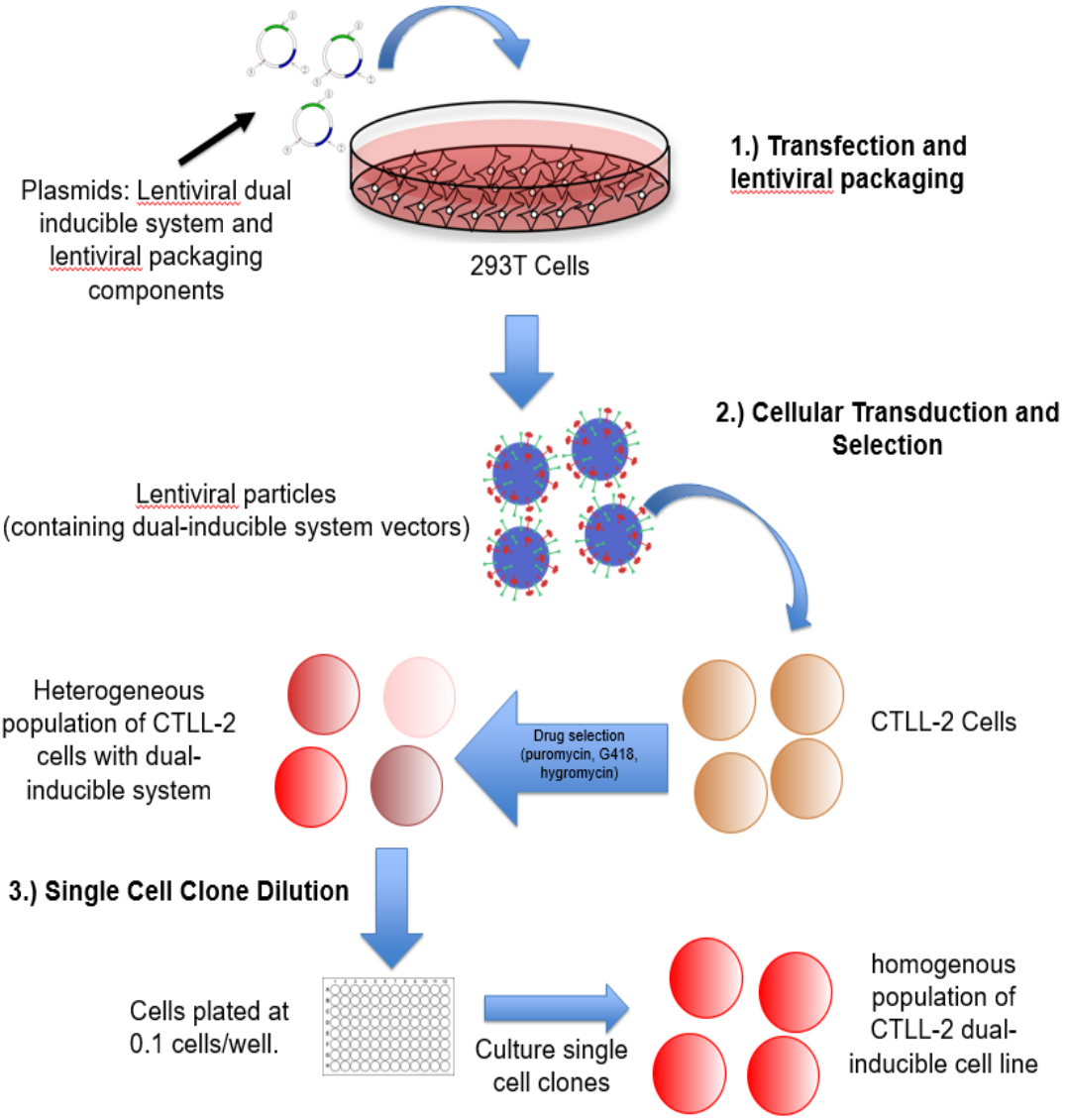

Figure 4. Creation of the dual-inducible CTTL-2 cell line.

infectious virus. Supernatant from these cells (containing secreted lentivirus) was collected and concentrated using ultracentrifugation over a sucrose gradient. Infectious lentiviral particles were then added to CTTL-2 cells and a spin inoculation was performed to ensure efficient transduction of target cells. Transduced cells were treated with antibiotic selection (hygromycin, G418, puromycin) after 3 days to select for cells with the dual-inducible integrated DNA vectors. A heterozygous population of CTTL-2 cells was then cultured containing the newly added dual-inducible system. Genetic variation was minimized through a single-cell isolation technique that cultures a homogenous cell line from a single CTTL-2 clone.

RESULTS

Induction of the dual-inducible CTLL-2 cell line

Two common molecular biology techniques used to verify induction of the dual-inducible expression include reverse transcriptase PCR (RT-PCR), a semi-quantitative method to quantitate RNA levels, and western blot analysis, which detects the level of a particular protein of interest (Figure 5).

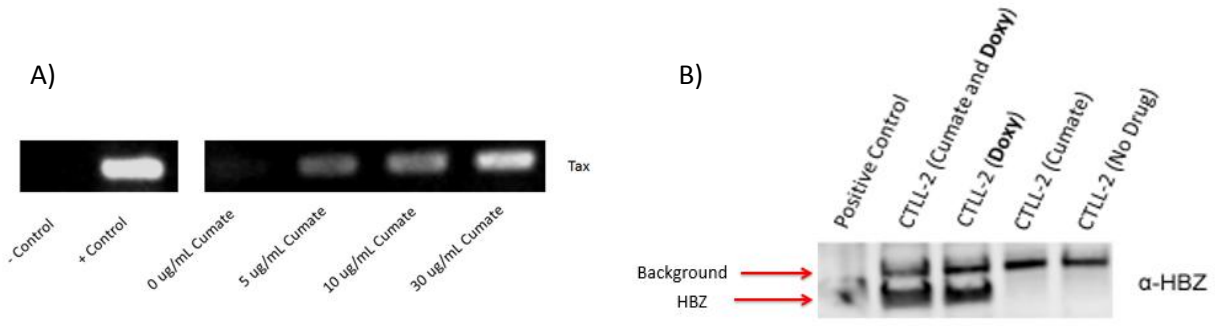

Figure 5. Verification of the dual-inducible expression. A) CTLL-2 dual-inducible cells were treated with titrating amounts of cumate for 24h. Total RNA was isolated followed by reverse transcription and PCR analysis of *tax* gene expression. B) CTLL-2 dual-inducible cells were treated with 30 µg/mL of cumate and 100 ng/mL of doxycycline, where indicated, for 24h. Cells were lysed and protein expression was examined by immunoblot.

Testing Tax and HBZ functionality in dual-inducible cells

To verify the functionality of the dual-inducible gene expression we utilize a luciferase-based reporter gene assay (Figure 6). Tax associates with and activates transcription from the viral LTR promoter through interactions with cellular transcription factors, such as CREB. HBZ represses Tax-mediated LTR-activation by competitive binding to these same cellular transcription factors. Therefore, we can use an LTR-luciferase reporter construct to measure both Tax-activation and HBZ-mediated repression of Tax activity:

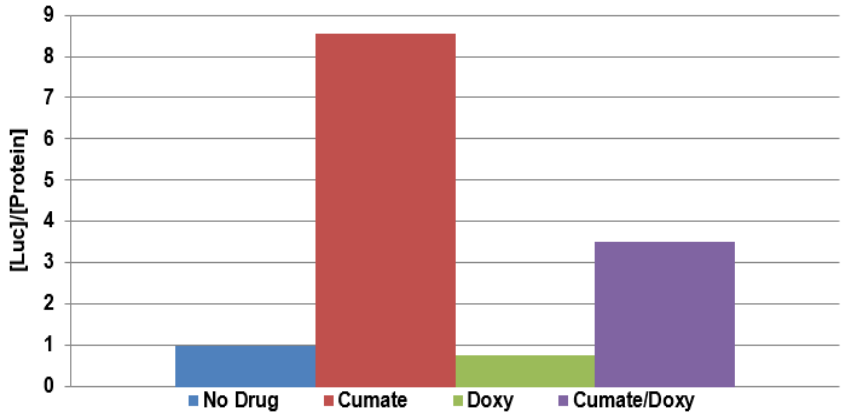

Figure 6. Relative LTR-luciferase activity in CTLL-2 dual-inducible cells. Dual-inducible CTLL-2 cells were treated with 30 µg/mL of cumate and 100 ng/mL of doxycycline, where indicated, for 24h. Cells were lysed in passive lysis buffer and firefly luciferase activity was measured using a luminometer. LTR-driven luciferase activity is shown relative to protein concentration. Addition of cumate (Tax) activates LTR-luciferase activity, while addition of doxycycline and cumate (Tax and HBZ) repress LTR-luciferase activity (compared to cumate alone).

Cellular viability in the presence of Tax and HBZ

CTLL-2 cells require addition of exogenous IL-2 to proliferate and remain viable in tissue culture. Continual growth in the absence of IL-2 is indicative of cellular transformation in CTLL-2 cells. We can use this property to measure the effects of Tax and HBZ on cellular transformation. This process involves the removal of IL-2 from media in tissue culture. Once IL-2 has been removed, we then plate equal numbers of

cells under varying concentrations of drug; Tax alone, HBZ alone, or Tax and HBZ. The number of live cells in each condition were enumerated daily (Figure 7). This method has previously been established and used by researchers in the field (Iwanaga et al. 1999).

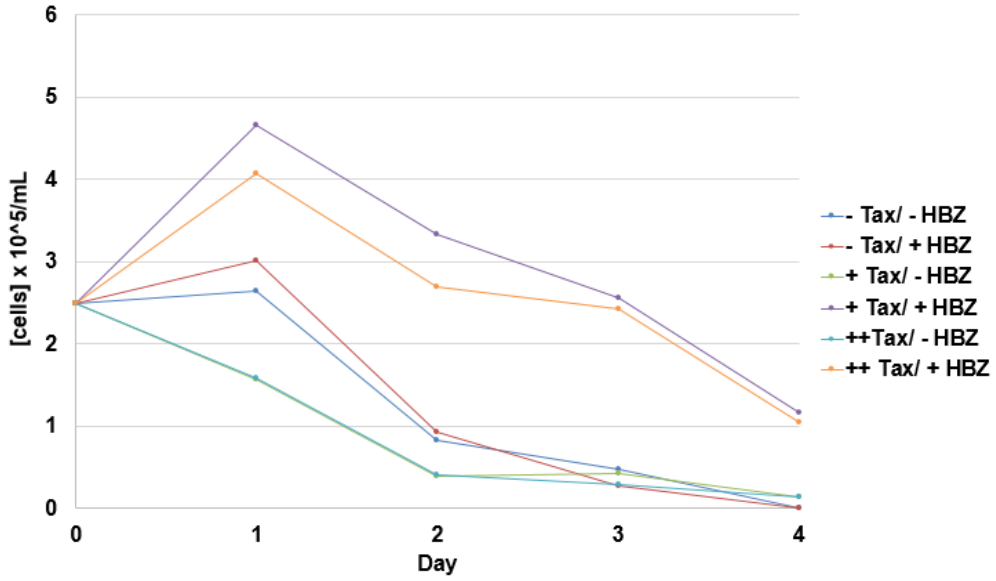

Figure 7. IL-2-independent transformation assay. CTLL-2 inducible cells were treated with 10 µg/mL of cumate (+), 30 µg/mL of cumate (++), and/or 100 ng/mL of doxycycline in the absence of IL-2. Live cells were counted daily using trypan blue exclusion method.

DISCUSSION

The literature has established the oncogenic potential of both Tax and HBZ, providing evidence to their contribution in ATLL development. However, in a newly infected cell the expression of multiple viral factors occurs concurrently and thus can influence one another. Therefore, we hypothesized that the interplay between Tax and HBZ ultimately enhances T-cell transformation and eventual ATLL development. To test this hypothesis, we used a dual-inducible lentiviral expression vectors that allows us to

control individual levels of Tax and HBZ within the cell. We successfully cloned Tax and HBZ cDNAs into cumate- and doxycycline-inducible lentiviral vectors, respectively (Figures 2-3). The dual-inducible lentiviral vectors were then transduced into CTLL-2 cells and stable cell lines were selected (Figure 4). Induction of Tax and HBZ in response to cumate and doxycycline was verified using RT-PCR analysis and immunoblotting (Figure 5). Functionality of the induced Tax and HBZ proteins was then further verified using an LTR-driven luciferase construct (Figure 6). Finally, our data has suggested that the presence of both Tax and HBZ enhanced cellular viability in CTLL-2 cells using our IL-2-independence transformation assay (Figure 7).

Future experiments will measure the levels of Tax and HBZ induced in CTLL-2 cells in response to titrating amounts of cumate and doxycycline. After this has been completed, we can then begin to compare these levels to HTLV-1-transformed cell lines. This will help us to determine the optimal range of cumate and doxycycline for use in our IL-2-independence transformation assays and ensure physiologic levels of Tax and HBZ. This will also enable us to examine any potential leakiness of our dual-inducible system. After fully characterizing the dual-inducible expression system, we can then begin to use optimized levels of cumate and doxycycline to generate IL-2-independent cell lines. These cell lines can then be tested for their dependence on Tax and/or HBZ by removing cumate and doxycycline. Microarray analysis will also be used to examine the cellular expression profile of IL-2-independent cell lines identifying the key cellular pathways involved in transformation. Ultimately, our work will provide better insight into the HTLV-1-mediated transformation landscape of T-cells and the molecular

pathogenesis of ATLL development with the ultimate goal to identify a therapeutic that targets critical pathways and will block cancerous cell growth.

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APPENDIX

Publications representing other work completed in the lab:

Panfil, A.R.; Dissinger, N.; **Howard, C.M**.; Murphy, B.; Landes, K.; Fernandez, S.; Green, P.L. 2016. Functional Comparison of HBZ and the Related APH-2 Protein Provide Insight into HTLV-1 Pathogenesis. *Journal of Virology*.

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